Notch-regulated ankyrin-repeat protein is a novel tissue biomarker that predicts poor prognosis in non-small cell lung cancer

YUFENG LIAO¹, JUNFENG CHEN¹, JIANBO MA¹, QIFENG MAO¹, RENXIONG WEI³ and JIANJUN ZHENG²

Departments of ¹Laboratory Medicine and ²Radiology, Ningbo No. 2 Hospital; ³Department of Clinical Medicine, Ningbo Municipal Hospital of Traditional Chinese Medicine, Ningbo, Zhejiang 315010, P.R. China

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Abstract. Notch-regulated ankyrin-repeat protein (NRARP) has recently been reported to be involved in a number of malignant cancers; however, its role in non-small lung cancer (NSCLC) remains unclear. The present study aimed to identify whether NRARP could be applied as a novel prognostic marker for NSCLC. A total of 108 NSCLC patients were enrolled in the present study and their lung tissues were collected. Reverse-transcription quantitative polymerase chain reaction and immunohistochemical staining were used to assess the mRNA and protein levels of NRARP. Appropriate statistical tests were performed to evaluate the associations between NRARP protein expression and clinicopathological features and prognosis in NSCLC patients. The results revealed that NRARP expression was significantly associated with tumor differentiation ($P=0.001$), Tumor-Node-Metastasis stage ($P=0.004$) and cigarette smoking ($P<0.001$). Furthermore, patients with higher NRARP protein expression had significantly shorter overall survival times ($P<0.001$). Multivariate analysis indicated that overexpression of NRARP protein could be applied as an independent prognostic biomarker for NSCLC. In summary, the present study demonstrated that NRARP protein is overexpressed in NSCLC and that high NRARP expression is correlated with tumor progression and overall survival time. These data indicated the potential value of NRARP as a novel therapeutic target for the treatment of NSCLC.

Introduction

Lung cancer is the most commonly diagnosed malignant tumor globally (1), and is currently the leading cause of cancer-associated mortality in China due to environmental pollution and a high prevalence of cigarette-smoking (2). According to histological types, lung cancers may be generally divided into small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC is the largest histological subtype, accounting for ~80% of all lung cancer cases (3). Despite advances in diagnosis and therapy over the last two decades, the overall survival (OS) rate for NSCLC patients remains at ~16% due to late-stage diagnosis and unsuccessful treatments (4). Consequently, there is an urgent demand for the detection of novel biomarkers that are capable of serving as diagnostic and prognostic markers for NSCLC.

Notch-regulated ankyrin-repeat protein (NRARP) is a negative feedback regulator in the Notch signaling pathway and is regulated by Notch protein (5). The overexpression of the Notch protein may promote NRARP expression; however, NRARP exerts feedback inhibition on the activity of Notch by inducing degradation of the Notch receptor intracellular domain (6). Previous studies have implicated Notch signaling and its downstream protein NRARP in the oncogenesis of various cancer types, including colorectal cancer (CRC) (7), thyroid (8), breast (9) and liver cancer (10). However, the role that NRARP serves in NSCLC remains unclear.

In the present study, the mRNA and protein expression levels of NRARP in NSCLC patients were investigated, and the associations of NRARP expression with clinicopathological characteristics and patient prognosis were explored.

Materials and methods

Tissue specimen selection and preparation. In the present study, the cancer tissues and paired adjacent tissues were collected from a total of 108 patients (74 males, 34 females) who underwent surgical resection of a NSCLC at Ningbo No. 2 Hospital (Ningbo, China) between January 2004 and June 2015. These patients received neither chemotherapy nor radiotherapy prior to surgery. The age of the selected patients ranged from 32 to

Correspondence to: Professor Jianjun Zheng, Department of Radiology, Ningbo No. 2 Hospital, 41 Northwest Street, Ningbo, Zhejiang 315010, P.R. China
E-mail: zhengjjmed@126.com

Dr Renxiong Wei, Department of Clinical Medicine, Ningbo Municipal Hospital of Traditional Chinese Medicine, 819 North Liyuan Road, Ningbo, Zhejiang 315010, P.R. China
E-mail: weirxmed@163.com

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74 years (mean, 54 years). Among these patients, 58 had squamous cell cancer (SCC) and 50 had adenocarcinoma (AC). The clinicopathological characteristics of the patients analyzed in the present study are shown in Table I. Tumor stage was classified according to the current International Union Against Cancer Tumor-Node-Metastasis (TNM) classification guidelines (11). Follow-up was conducted by telephone interviews until December 2016 (median follow-up duration, 48.2 months; range, 4-110 months). OS was defined as the time interval between tumor resection and mortality. Written consent forms were obtained from each patient prior to sample collection. Ethical approval for this study was obtained from the Ethics Committee of Ningbo No. 2 Hospital. Fresh lung tissues from 40 patients were snap-frozen in liquid nitrogen and stored at -80°C for reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis. For immunohistochemistry (IHC) experiments, tissues from all 108 patients were cut into 1x1-cm cubes and immersed in 4% paraformaldehyde.

RNA extraction and RT-qPCR. Total RNA samples from fresh primary NSCLC tissues from 40 patients (20 SCC, 20 AC) and their corresponding normal tissues were extracted using RNAiso Plus® (Takara Biotechnology, Co. Ltd., Dalian, China). The concentrations of RNA in the samples were determined by reading the absorbance at 260 nm. RT of total RNA (1 μg) was performed with a PrimeScript® RT Master Mix (Perfect Real-Time) kit (Takara Biotechnology, Co. Ltd.) according to the manufacturer's protocol (37°C for 15 min, 85°C for 5 sec and then maintained at 4°C). The resulting cDNA was then amplified using a SYBR® Premix Ex Taq™ II (Perfect Real-Time) kit (Takara Biotechnology, Co., Ltd.) with an ABI 7500 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primers used were as follows: NRARP forward, 5'-ATCTTCCAGGAGGCTGTGC-3'; NRARP reverse, 5'-CTTCGCCTTGGTGATGAGAT-3'; GAPDH (internal control) forward, 5'-CCCTTCATTGACCTCAACTAC-3'; and GAPDH reverse, 5'-CCACCTTCTTGATGTCATCAT-3'. The conditions used for PCR were as follows: 30 sec incubation at 95°C ; followed by 40 cycles of 95°C for 5 sec and 64°C for 34 sec. The $2^{-\Delta\Delta\text{C}_q}$ method was applied to analyze gene expression (12). For each gene, three independent experiments were performed.

IHC analysis. The paraformaldehyde-fixed, paraffin-embedded tissue blocks were cut into 4- μm thick sections. Hematoxylin and eosin staining was performed to ensure the inclusion of normal or tumor cells. Subsequently, IHC staining was performed to detect the NRARP protein expression in lung tissues. To summarize, sections were initially baked at 65°C for 30 min and then deparaffinized in xylene and rehydrated in a series of ethanol solutions with increasing concentration. Next, the sections underwent antigen retrieval in a microwave for 10 min, and 3% H_2O_2 -methanol was used to block endogenous peroxidase activity. Following blocking of nonspecific staining with normal goat serum, sections were incubated overnight at 4°C with rabbit anti-human NRARP polyclonal antibodies (dilution, 1:100; cat. no. HPA025729; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany); then, sections were washed in phosphate-buffered saline (PBS) three times and incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG

(H+L) secondary antibody (dilution, 1:500; cat. no. A0208, Beyotime Institute of Biotechnology, Haimen, China) for a further 30 min at room temperature. Finally, sections were developed with 3,3-diaminobenzidine solution (cat. no. D3939; Sigma-Aldrich, Merck KGaA) according to the manufacturer's protocol at room temperature for 20 sec, and were then counterstained with 0.1% hematoxylin at room temperature for 30 sec. For the negative control, the tissues were incubated with PBS instead of the primary antibody. Breast cancer tissues known to have a high expression of NRARP were used as the positive control. The breast cancer tissues were collected from 12 patients who underwent surgical resection of a breast cancer at Ningbo No. 2 Hospital. Written consent forms were obtained from all patients.

IHC scoring. The NRARP expression levels in the sections were examined by light microscopy. NRARP staining in tumor and normal tissues was scored semi-quantitatively according to the intensity of staining and the percentage of positively stained cells. The overall immunoreactivity score (IRS) was calculated as the sum of the staining intensity score (0, no staining; 1, mild staining; 2, moderate staining; 3, strong staining) and the score for the percentage of positively stained cells (0, <10%; 1, 11-25%; 2, 26-50%; 3, 51-75%; 4, >75%). According to the IRS, each tissue was defined as having low (score 0-3) or high (score 4-7) NRARP expression. Two independent histopathologists, who had no knowledge of the clinicopathological information, evaluated the sections.

Statistical analysis. The data were presented at the mean $\pm$ standard deviation. A one-way ANOVA (with Student-Newman-Keuls as a post hoc test) or Student's t-test was used to compare continuous variables. The Pearson χ^2 test was used to assess the association between NRARP expression and pathological parameters. The Kaplan-Meier method was used to estimate the OS, and significance was assessed using log-rank test. The Cox proportional hazards regression model was used for univariate and multivariate analyses. All P-values corresponded to two-sided tests and $P < 0.05$ was considered to indicate a statistically significant difference. All statistical calculations were performed by using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) statistical software.

Results

NRARP expression in NSCLC patients. In order to investigate oncogenic properties of NRARP in NSCLC, the expression of NRARP was assessed by IHC in 108 tumor tissues and adjacent normal tissues from NSCLC patients. Fig. 1A demonstrates that NRARP expression was significantly increased in the tumor tissues, and the staining was predominantly located in the cytoplasm of tumor cells. The percentage of tumor tissues exhibiting high expression of NRARP was 52.78% (57/108) among all patients, 51.72% (30/58) in patients with SCC, and 54.00% (27/50) in patients with AC. By contrast, only 5.56% (6/108) of normal tissues exhibited high NRARP expression. In addition, compared with their respective normal tissues, SCC and AC tissues each had a significantly higher IRS (Fig. 1B). Subsequently RT-qPCR was used to assess NRARP mRNA expression in fresh tumor and normal tissues collected from 40

Table I. Associations between NRARP protein expression and clinicopathological features in patients with NSCLC.

Clinical features	Cases, n	NRARP expression, n (%)		P-value
		Low	High	
Tissue type				<0.001
Normal lung	108	102 (94.4)	6 (5.6)	
NSCLC	108	51 (47.2)	57 (52.8)	
Sex				0.420
Male	74	33 (44.6)	41 (55.4)	
Female	34	18 (52.9)	16 (47.1)	
Age, years				0.607
≤50	43	19 (44.2)	24 (55.8)	
>50	65	32 (49.2)	33 (50.8)	
Histological type				0.803
SCC	58	28 (48.3)	30 (51.7)	
AC	50	23 (46.0)	27 (54.0)	
Differentiation				0.001
Well	42	29 (69.0)	13 (31.0)	
Moderate	42	13 (31.0)	29 (69.0)	
Poor	24	9 (37.5)	15 (62.5)	
TNM stage				0.004
I	40	27 (67.5)	13 (32.5)	
II	42	16 (38.1)	26 (61.9)	
III	26	8 (30.8)	18 (69.2)	
T classification				0.847
T1, T2	54	26 (48.1)	28 (51.9)	
T3, T4	54	25 (46.3)	29 (53.7)	
N classification				0.974
N0	57	27 (47.4)	30 (52.6)	
N1, N2, N3	51	24 (47.1)	27 (52.9)	
Cigarette smoker				<0.001
Yes	73	26 (35.6)	47 (64.4)	
No	35	25 (71.4)	10 (28.6)	

NRARP, Notch-regulated ankyrin-repeat protein; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; AC, adenocarcinoma; TNM, Tumor-Node-Metastasis.

patients with NSCLC. Fig. 1C demonstrates that the NRARP mRNA levels in SCC and AC tissues were markedly increased compared with their adjacent normal tissues. Taken together, these results suggest that the mRNA and protein expression levels of NRARP are elevated in NSCLC tissues and the over-expression of NRARP was observed in all NSCLC cells.

Association between NRARP expression and pathological features of NSCLC. The associations between NRARP expression levels and the clinicopathological features (including sex, age, histological type, differentiation, TNM stage, T classification, N classification and smoking status) of patients with NSCLC were analyzed (Table I). The results demonstrated

that increased NRARP protein expression was significantly associated with differentiation ($P=0.001$), TNM stage ($P=0.004$) and the smoking status of the patient ($P<0.001$). By contrast, no significant association was identified between high NRARP protein expression and sex ($P=0.420$), age ($P=0.607$), histological type ($P=0.803$), T classification ($P=0.847$) or N classification ($P=0.974$).

Survival analysis of NRARP expression in NSCLC. The prognostic significance of high NRARP expression and the survival curves in NSCLC patients were determined by using the Kaplan-Meier method. The results showed that aberrant high expression of NRARP in NSCLC patients was significantly associated with shorter OS time ($P<0.001$, Fig. 2A). Additionally, the same association was identified in SCC and AC patients ($P<0.001$, Fig. 2B and C). Univariate analysis demonstrated that NRARP expression, differentiation, TNM stage, T classification, N classification and cigarette smoking were associated with the prognosis of patients with NSCLC (Table II). Multivariate analysis identified that NRARP expression ($P<0.001$), differentiation ($P=0.006$) and TNM stage ($P=0.001$) were all independent prognostic markers for OS in NSCLC patients (Table II). Taken together, these data suggest that NRARP may be applied as a valuable biomarker for predicting prognosis in NSCLC patients.

Discussion

In the present study, the results demonstrated that NRARP expression was increased in human NSCLC tissues at the mRNA and protein levels. In addition, high NRARP expression was associated with tumor differentiation, TNM stage and whether the patients were cigarette smokers. Furthermore, it was identified that patients with NSCLC with high NRARP expression had a significantly shorter OS time compared with patients with low NRARP expression. Multivariate analysis revealed that NRARP was an independent prognostic factor for OS in NSCLC patients. Collectively, these results suggested the potential value of NRARP as a novel biomarker for the prediction of NSCLC prognosis.

The Notch signaling pathway participates in a plethora of cell activities, including cell proliferation, differentiation and apoptosis. Although its specific role in tumorigenesis remains controversial, it has been reported that dysregulation of Notch signaling is associated with various types of cancer, including CRC and breast, prostate, bladder and thyroid cancers (13-17). NRARP, a downstream effector of Notch signaling, is a small, evolutionarily conserved protein containing two ankyrin-repeat motifs (5). Previous studies have suggested a close and complex association between Notch signaling and NRARP (5,18). Because NRARP acts as a downstream target of Notch signaling, its expression may be regulated by Notch signaling. Conversely, NRARP may also function as a negative feedback regulator of Notch signaling and suppress the activation of Notch signaling by promoting the degradation of Notch intracellular domain (19). A previously study demonstrated that NRARP was upregulated in thyroid cancer in response to over-activated Notch signaling and that downregulation of NRARP may inhibit thyroid cancer cell proliferation, induce G_1 arrest, inhibit cell invasion, and

Figure 1. NRARP expression in NSCLC and adjacent normal lung tissues. (A) Representative images of immunohistochemical staining for NRARP (original magnification, x200; hematoxylin counterstaining). (B) IRS of NRARP protein expression in normal lung tissue, SCC and AC. (C) NRARP mRNA expression in normal lung tissue, SCC and AC. ** $P < 0.001$ compared with normal lung tissue. NRARP, Notch-regulated ankyrin-repeat protein; NSCLC, non-small cell lung cancer; IRS, immunoreactive score; SCC, squamous cell carcinoma; AC, adenocarcinoma.

Figure 2. Kaplan-Meier survival analysis of NSCLC patients with high NRARP vs. low NRARP expression. (A) The overall survival difference between patients with high and low NRARP expression. (B and C) Overall survival curves for high vs. low expression of NRARP in (B) SCC and (C) AC patient subgroups. P-values were calculated by log-rank test. NSCLC, non-small cell lung cancer; NRARP, Notch-regulated ankyrin-repeat protein; SCC, squamous cell carcinoma; AC, adenocarcinoma.

promote apoptosis (8). In breast cancer, the Notch pathway was revealed to be dysregulated, and silencing of NRARP in human breast cancer cell lines led to reduced cell growth (9).

These studies supported the hypothesis that NRARP serves a direct oncogenic role in connecting Notch signals to cancer progression.

Table II. Univariate and multivariate statistical analyses for various prognostic parameters in patients in NSCLC.

Characteristic	No.	Univariate analysis		Multivariate analysis		
		P-value	Regression coefficient (SE)	P-value	Relative risk	95% CI
Sex		0.132		-	-	-
Male	74					
Female	34					
Age, years		0.465		-	-	-
≤50	43					
>50	65					
Histological type		0.066		-	-	-
SCC	58					
AC	50					
Differentiation		<0.001	1.012 (0.179)	0.006	1.780	1.176-2.694
Well	42					
Moderate	42					
Poor	24					
TNM stage		<0.001	0.974 (0.138)	0.001	1.832	1.272-2.640
I	40					
II	42					
III	26					
T classification		0.018	0.551 (0.233)	0.151	n.s.	n.s.
T1, T2	54					
T3, T4	54					
N classification		0.029	0.507 (0.232)	0.471	n.s.	n.s.
N0	57					
N1, N2, N3	51					
Cigarette smoker		0.012	0.653 (0.261)	0.539	n.s.	n.s.
Yes	73					
No	35					
NRARP expression		<0.001	1.337 (0.262)	<0.001	2.975	1.779-4.975
Low	51					
High	57					

NSCLC, non-small cell lung cancer; SE, regression coefficient; CI, confidence interval; SCC, squamous cell carcinoma; AC, adenocarcinoma; TNM, Tumor-Node-Metastasis; NRARP, Notch-regulated ankyrin-repeat protein; n.s., non-significant.

At present, the majority of patients with NSCLC are diagnosed at advanced stages, which leads to unsatisfactory prognosis. The traditional factors for evaluating prognosis, such as TNM stage, lymph node status and histological differentiation, at present, do not meet the developing demands for accurate and individualized prognostic evaluation in patients with NSCLC. Thus, there is an urgent requirement to identify novel biomarkers to better predict the prognosis these patients.

Several studies have revealed that NRARP is associated with prognosis in different types of cancer. For example, Chu *et al* (8) demonstrated that NRARP was highly expressed in thyroid carcinoma compared with normal thyroid tissues, and also identified that NRARP protein level was negatively associated with patient prognosis. Similar results were obtained in breast cancer, wherein high NRARP expression was found to be associated with shorter survival time (9). However,

decreased NRARP expression in CRC tissues compared with normal intestinal epithelium has been reported, and significantly longer survival times were observed in such patients with high NRARP expression (7).

To the best of our knowledge, the present study is the first to explore the prognostic value of NRARP in NSCLC. The data demonstrated that NRARP protein expression was associated with tumor differentiation, TNM stage and smoking status. In addition, patients with NSCLC who had a higher NRARP expression had a worse prognosis, which was consistent with the aforementioned results in other types of tumor. All of these findings suggest that NRARP has the potential to serve as an independent biomarker for the prognosis of NSCLC.

Presently, there are only a small number of studies that have been published regarding the functions of NRARP in tumorigenesis. Several genetic observations suggest that functional

crosstalk exists between WNT and Notch signaling (20) and NRARP may participate in tumorigenesis through this crosstalk. In human breast cancer, *NRARP* may serve a functional role in the 'rewiring' of Notch and WNT pathways, in which incoming Notch signals from neighboring cells lead to expression of WNT target genes in breast cancer cells (9). Furthermore, it has been demonstrated that Notch signaling may directly target NRARP and suppress the expression of WNT target genes in human CRC cells through epigenetic modification (7). Additionally, in a previous study, treatment with Lenti-NRARP-shRNA in thyroid cancer cells was able to significantly suppress matrix metalloproteinase 9 expression and inhibit thyroid cancer cell invasion, which suggested that downregulation of NRARP promotes the activation of Notch, subsequently inhibiting WNT signaling and cell invasion (8).

Epithelial-mesenchymal transition (EMT) is a vital pathological mechanism in the progression of the majority of tumors. The research conducted by Zhu *et al* (10) verified that NRARP knockout leads to attenuation of cancer cell stemness, which is linked with EMT. Furthermore, two EMT-associated transcription factors were also revealed to be highly associated with NRARP (21,22). Additionally, Fazio *et al* (23) demonstrated that increased NRARP was involved in NOTCH-induced EMT in CRC, and also enhanced the functional association with NRARP and EMT. However, no research regarding the mechanism of NRARP in NSCLC has been reported and this consequently requires elucidation by further studies.

In conclusion, the present study demonstrated that NRARP is aberrantly expressed in NSCLC and that high NRARP protein expression is associated with tumor progression and OS. These results indicated the potential value of NRARP as a novel therapeutic target for the treatment of NSCLC. However, further studies are required in order to determine the definitive role that NRARP serves in NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL and JC performed the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. JM conducted the human studies. QM performed the statistical analysis. RW and JZ conceived the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written consent forms were obtained from each patient prior to sample collection. Ethical approval for this study was obtained from the Ethics Committee of Ningbo No. 2 Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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